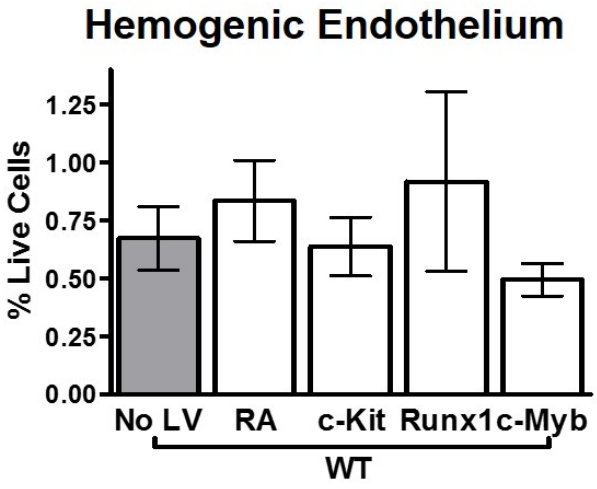
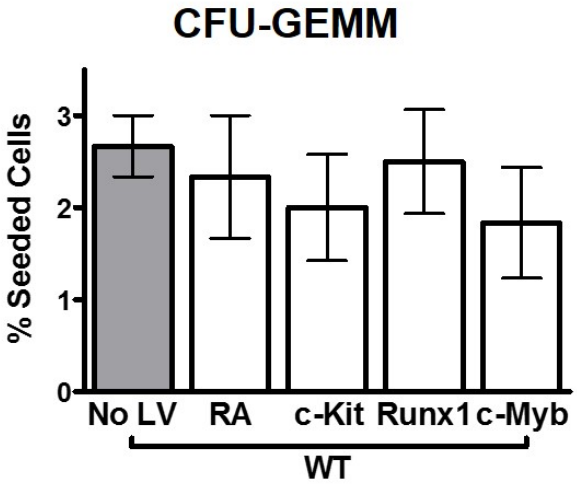

Hemogenic Endothelium
% Live Cells
1.25
1.00
0.75
0.50
0.25
0.00
No LV
RA
c-Kit
Runx1
c-Myb
WT


CFU-GEMM
% Seeded Cells
3
2
1
0
No LV
RA
c-Kit
Runx1
c-Myb
WT

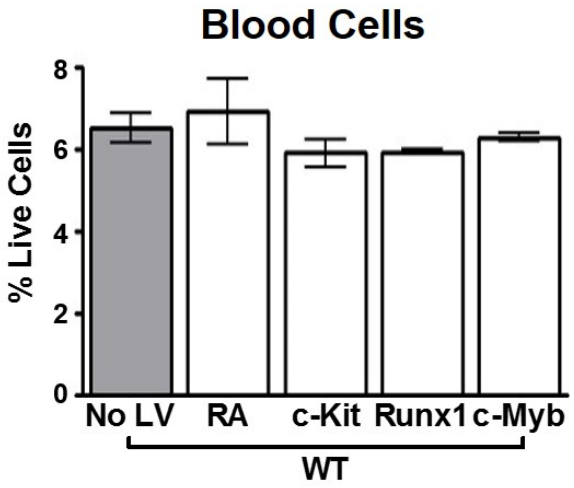

Blood Cells
% Live Cells
8
6
4
2
0
No LV
RA
c-Kit
Runx1
c-Myb
WT

Figure S1. Lentivirus-infected wildtype yolk sacs do not demonstrate ectopic hemogenic endothelial cell specification or hematopoiesis. (Related to Figure 3) **A.** Quantitative analysis of $Flk1^{+}c\text{-}Kit^{+}CD45^{-}$ SP hemogenic endothelial cells from untreated (No LV), RA-treated (RA), and lentivirus-infected *Raldh2*$^{+/+}$ (WT) yolk sacs. Data points were calculated as a percentage of total live cell population ± SEM (n ≥ 3). **B.** Total multilineage CFU-GEMM colonies generated from hemogenic endothelial cells, calculated as percentage of seeded cells (100 cells per well) ± SEM (n ≥ 3). **C.** Quantitative analysis of $Flk1^{-}c\text{-}Kit^{+}CD45^{+}$ Non-SP blood cells from untreated (No LV), RA-treated (RA), and lentivirus-infected *Raldh2*$^{+/+}$ (WT) yolk sacs. Data points were calculated as a percentage of total live cell population ± SEM (n ≥ 3). Statistical analysis of significance was determined by Student's t test with a confidence interval of 95% ($p \leq 0.05$).

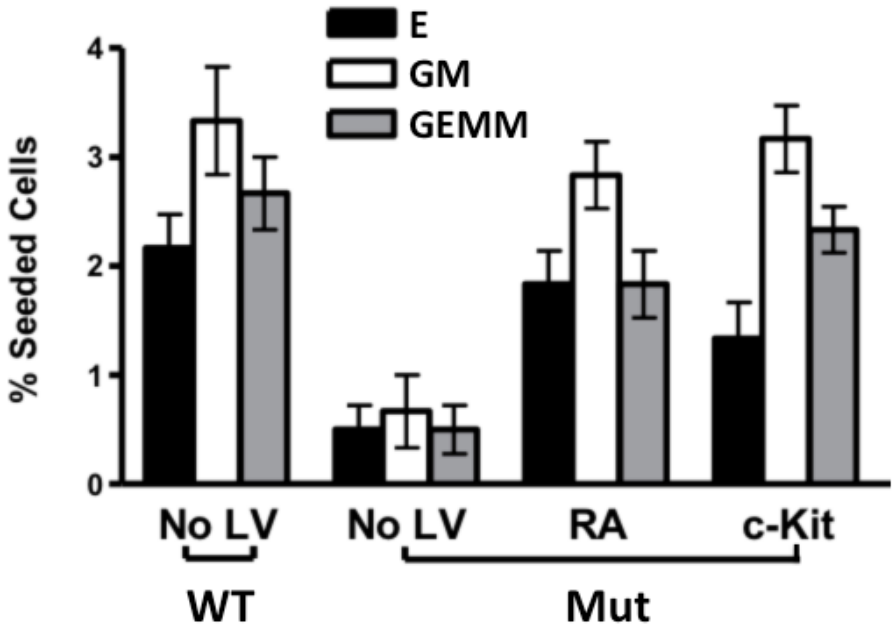


Figure S2. Individual colony types as scored from methylcellulose assay for all treatment groups. (Related to Figure 3) Erythroid (E), granulocyte-macrophage (GM), and granulocyte-erythroid-macrophage-megakaryocyte (GEMM) colony-forming units were scored separately. Total number of colonies generated are reported per 100 seeded cells ± SEM (n ≥ 3). *p* values following Student's t test with a confidence interval of 95% (p ≤ 0.05) are as follows for **E colonies**: ***No LV WT vs. No LV Mut, p = 0.001***; No LV WT vs. RA Mut, p = 0.461; No LV WT vs. c-Kit Mut, p =0.096; ***No LV Mut vs. RA Mut, p = 0.006***; No LV Mut vs. c-Kit Mut, p = 0.065. *p* values following Student's t test with a confidence interval of 95% (p ≤ 0.05) are as follows for **GM** colonies: ***No LV WT vs. No LV Mut, p = 0.001***; No LV WT vs. RA Mut, p = 0.411; No LV WT vs. c-Kit Mut, p =0.781; ***No LV Mut vs. RA Mut, p = 0.001***; ***No LV Mut vs. c-Kit Mut, p < 0.001***.

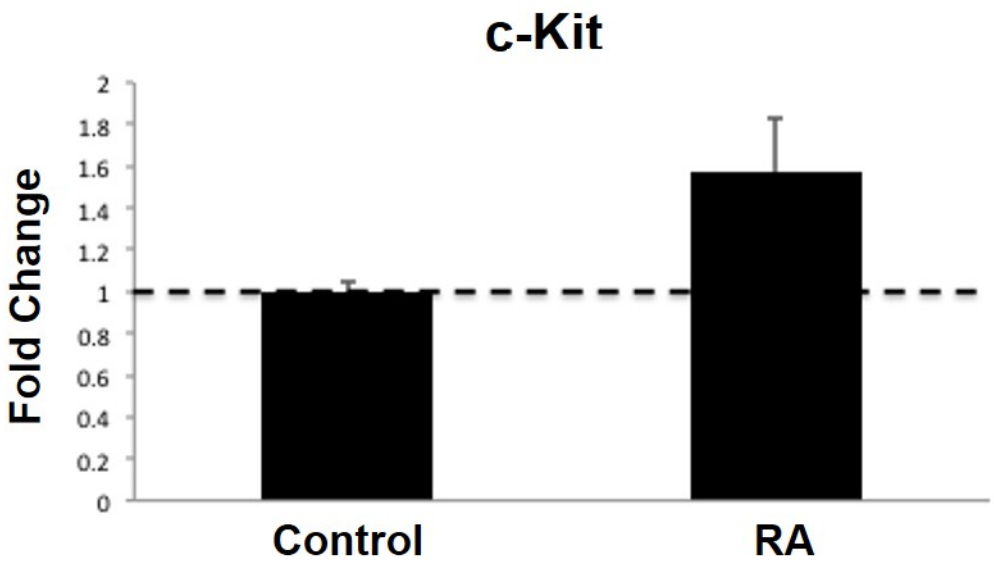


Figure S3. Retinoic acid regulation of c-Kit epression (Related to Figure 4). Human umbilical vein endothelial cells (HUVEC), which uniquely express low levels of c-Kit under control conditions, were treated with exogenous all-trans retinoic acid (RA) at a concentration (0.5 μM) known to inhibit endothelial cell cycle progression (Lai et al., 2003). Gene expression was analyzed via qPCR and calculated relative to endogenous β-actin expression; data represent mean ± SEM (n ≥ 3). Relative to untreated controls, RA treatment increased c-Kit protein expression by ~1.6-fold within 24 h, as measured via flow cytometry.

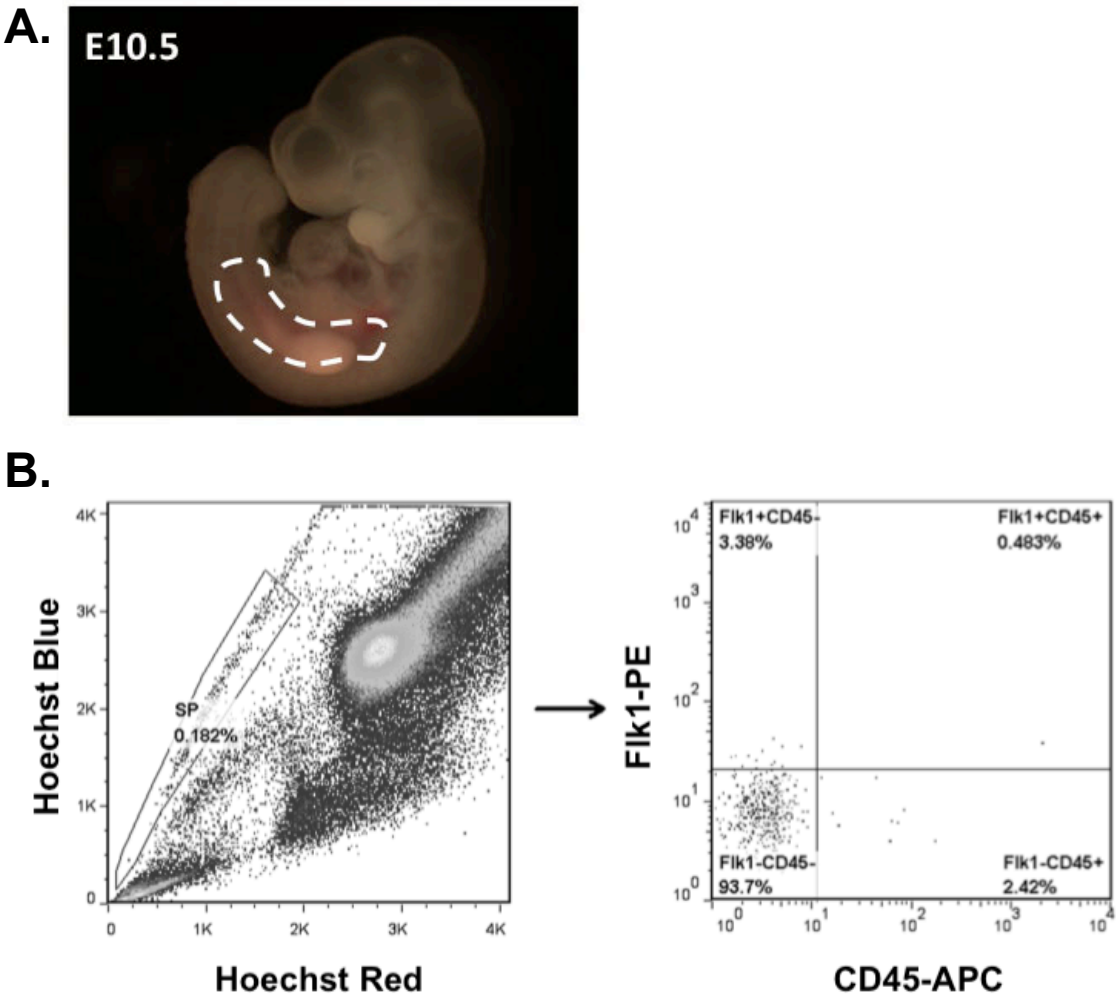


Figure S4. Flk1 and CD45 are mutually exclusive within the E10.5 AGM side population. (Related to Figure 7) **A.** E10.5 wildtype embryo, with dotted lines representing AGM region, which was microdissected for cell sorting and FACS analysis. **B.** Representative profile from FACS analysis of Flk1 and CD45 expression within the SP, which demonstrates virtually no co-expression of these markers (0.48%) within the SP population.

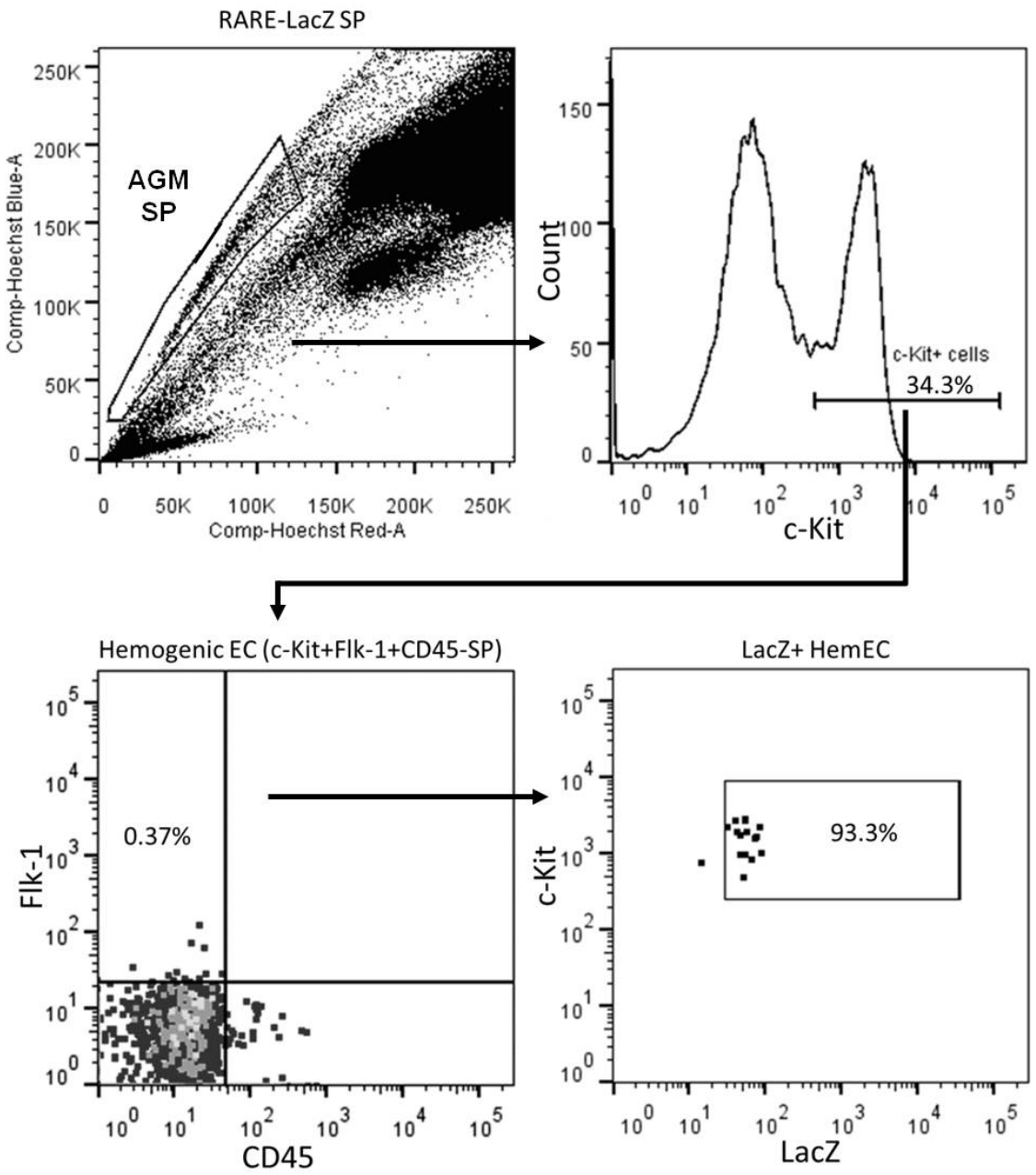


Figure S5. Hemogenic endothelial cells from E10.5 AGM are RA-responsive. (Related to Figure 7) FACS analysis of Flk1^{+}c-Kit^{+}CD45^{-} SP cells from E10.5 *Raldh2*$^{+/+}$; RARE-*lacZ* AGM tissue demonstrate that 93.3% of hemogenic endothelial cells are undergoing active RA signaling.